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13. ABSTRACT (Maximum 200 words)

As a first step in using nematocysts isolated from Physalia in a drug-delivery patch, we needed to assess the techniques used to yield efficient discharge. A number of pre-discharge and discharge solutions were utilized to determine the best combination, chosen based on literature findings for nematocysts isolated from other species. Note that Physalia is not a true jellyfish, suggesting that the mechanisms behind discharge are not exactly the same. The next step was to examine the puncture mechanics of the thread, by assessing the ability of the thread to puncture materials with a range of elastic modulus ranging from 0.02 to 90,000 MPa. The puncture tests were accomplished using excised tentacles from Physalia as a best-case scenario due to physical immobilization of the nematocysts as well as physiological discharge induced by mechanical stimulation of the cnidocyte cnidocil. To date, the puncture mechanics of the thread have only been theoretically calculated using mass and velocity information. Additionally, lectin-binding studies were conducted to examine the potential for using the lectins as a means for immobilization. Lectins are sugar-binding proteins that bind to moieties present on the surface of nematocysts. Finally, optical tweezing was used to evaluate the manipulation of individual nematocysts as a means for fabricating the patch.

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Project B: Sequence the Transcriptome of the Cnidocyte

The aims of this phase of the project are two-fold. First, to characterize the transcriptome of developing cnidocytes and, thereby, obtain an inventory of all the genes involved in the creation of cnidocytes, particularly those involved in the generation and packaging of toxins into the cysts. Second, to establish a cell line for cnidocyte precursor cells, thereby providing the means to obtain a ready supply of cnidocytes. Together, these two components will enable us to produce cnidocytes, and hence cysts, that produce desirable therapeutic agents instead of toxins.

Milestone 2: Sequence the transcriptome

This phase involves generating and sequencing amplified cDNA libraries for developing cnidocytes. Our approach has been to create two separate libraries: one for cnidocytes alone, the other for whole tentacles.

In the Portuguese Man O'War, *Physalia,* cnidocytes develop in the bulbous dactylozooid, which is found at the base of each tentacle. The cnidocyte undergoes near complete development in the dactylozooid, and then migrates into the base of the growing tentacle. For the isolated cnidocyte libraries, therefore, we excised intact tentacles, together with the basal dactylozooid. Together, these tissues will provide the complete developmental profile of the cnidocyte, from the most immature to the fully mature stages. These were then dissociated using heat shock. Cnidocytes were isolated from other cells in the resulting homogenate using density centrifugation through Percol, a step that takes advantage of the density of the cyst in the cnidocyte. RNA was then obtained from the purified cnidocytes.

The whole tentacle libraries, which was prepared from dactylozooids and their adjacent tentacle bases, were created under the rationale that even though they would contain transcript that were specific for cells other than cnidocytes (i.e. nerve, muscle) they would likely provide a population of cnidocytes RNAs that might be better quality (i.e. longer or even complete fragments) than that isolated from purified cnidocytes, thereby making it easier to obtain full length sequence for transcripts of interest for our understanding of the composition and development of cnidocytes.

RNA isolation

Conventional RNA isolation methods, which work well for mammals and a variety of other organisms, works poorly, if not at all, on many cnidarians. This is probably because these animals produce large amounts of mucus (mucopolysaccharides), which traps the RNA, thereby preventing its purification. This has not proved to be a particular problem with isolated cnidocytes, possibly because the cnidocytes are separated from the mucus during the density centrifugation step, but was a persistent problem with whole tentacles.

We found, however, that we can obtain high quality RNAs from tentacles and, indeed all cnidarian species examined to date, using an RNA isolation method developed for dry seeds of the black spruce (Tai et al., 2004 Plant Molecular Bio Reporter 22, 93a-93e), which also contain large amounts of polysaccharide.

Briefly, excised tentacles or pellets of purified cnidocytes were transferred to a 1.5 ml Eppendorf tube and, homogenized in 0.5 ml Urea-$LiCl_2$ solution with a 1.5 pellet pestle. The homogenate was kept overnight at 4EC. The lysate was then added to the top of a Quiashredder spin column and centrifuged at 5,000g, for 30 min at 4EC. The pellet was resuspended in 0.5 ml resuspension buffer and centrifuged at 20,000g for 5 min. Two phenol/bromochloropropane extractions were performed on the resuspended pellet to segregate RNAs from other contaminants (proteins and DNA). RNAs present in the aqueous phase is then precipitated in 2 volumes of 2% potassium acetate in 95% ethanol and kept at -20EC overnight. RNA was collected by centrifugation at 25,000g, for15 min at 4EC. The pellet was washed with 70 %

ethanol, air dry and resuspended in 20-50 μL of DEPC-water. RNA quality was assessed by capillary electrophoresis using an Agilent 2100 Bioanalyzer.

Construction of EST libraries

Amplified cDNAs were prepared from 500 ng total RNA using the Marathon cDNA amplification kit (Cat# 634913, BD Biosciences, Clontech) with the following modifications. The primer used for the first-strand synthesis was replaced by the oligo(dT) primer, TRSA: CGCAGTCGGTACTTTTTTTTTTTT (Matz, 2002). Purification and concentration of doubled-stranded cDNA was done using the DNAclear kit (Cat#1756, Ambion). The purified cDNA was then ligated to a double stranded adaptor made of the 2 following oligonucleotides: LU4 adaptor, CGACGTGGACTATCCATGAACGCAACTCTCCGACCTCTCACCGAGTACG and ST12, CGTACTCGGT. The ligation product was cleaned up and submitted to 15 cycles of amplification using LaTaq polymerase, reaction buffer and dNTP mix with 0.1 μM of each LU4 primer CGACGTGGACTATCCATGAACGCA and the TRSA primers. PCR conditions were as follow: denaturation at 94EC for 30 sec, annealing at 65EC for 1 min, and extension at 72EC for 2.5 min. Ten microliters of the PCR product was electrophoresed on a 1% agarose gel and amplified product greater than 1 Kb in size was gel extracted using a Gel extraction kit (Quiagen) and eluted with 30 μl water. A second PCR reaction of 10 cycles was performed using 7.5 μl of the first PCR product, under the same conditions as before. The PCR product was then ligated into pGEM-T vector (Promega) and incubated at least 3 days before being transformed in Top-10 competent cells according the manufacturer's protocol. One sixth of the volume of transformed cells were spread on selective LB bioassay dishes (cat#240845, Nunc) and incubated at 37EC overnight. Dishes were sent to the Univ. of Florida DNA Sequencing Core/ICBR for colony picking and sequencing.

Construction of 454 Library

This was done using proprietary methods developed by Dr. Leonid Moroz, a colleague at the Whitney Laboratory, and one of the leading developers of methodologies for high throughput sequencing. The computer for the large scale sequence alignment and bioinformatics analysis of the resulting contigs has been assembled and is operational.

Results.

While preliminary studies indicated that the purified cnidocyte and tentacle libraries were high quality (i.e. low redundancy, and broadly representative) further analysis of EST and 454 sequence data revealed that they both had unacceptably high levels (84%) of ribosomal RNA (rRNA). In the preliminary studies that formed the basis of the proposal for this project, the amount of rRNA in the amplified cDNA libraries obtained from isolated cnidocytes was reasonable and appropriate; the reason for the change in quality is unclear. Additional libraries were prepared using the same methods, but they too had unacceptably high levels of rRNA.

We then pursued strategies to correct this deficiency. First, the TRSA primer was replaced with two different oligo-dT primers, but the resulting libraries also had high levels of rRNA. While the levels of rRNA in these libraries is unaceptable, we were able to salvage some useful sequence information from them by selectively removing rRNA from the results. This yielded 10,944 EST sequences and a total of 103,000 454 sequences. The assembly of these sequences was subjected to a Paracel Blast program and produced 8031 hits. These can be found at the following site: http://helix2.biotech.ufl.edu:26980/bq/ username: christelle; password: bouchard.

New libraries were subsequently made from isolated mRNA instead of using total RNA,

using the TRSA primer protocol outlined above. Test runs on these libraries showed that they had acceptable levels of rRNA (7 – 11%) and the initial titration step produced fragments of average length of 260 base pairs (bp), which are more than adequate for 454 sequencing.

The 454 company is releasing a new kit this fall that will yield 400 – 500 bp fragments, which will make the task of aligning the fragments into full length transcripts far more successful. Conseequently, we will delay performing 454 full runs until the new protocol is up and running at the UF sequencing center.

Milestone 3. Interstitial Cell Lines for Cnidocytes:

We have also developed methods for culturing cells from jellyfish. The work began using *Physalia*, but has also used the sea nettle, *Chrysaora*, and the hydroid *Cladonema* to accommodate the seasonal availability of *Physalia*.

The bases of tentacles were excised from the bell of *Chrysaora*, rinsed in fresh sea water containing antibiotics and digested for one hour, at room temperature, in 1000U/ml collagenase. The digested tentacle fragments were then transferred, with two rinses, to fresh, antibiotic-containing, filtered sea water, then finally to culture medium (Domart-Coulon, et al., 2001. PNAS 98: 11885-11890) and triturated using a descending series of pipette diameters. The cell suspension was then transferred to culture plates treated with a variety of substrates. Cells in untreated plates survived for up to two months. During that time they did not attach to the untreated substrate but, nevertheless, increased in cell number (Figure 2).

A survey of the literature revealed that conventional tissue culture substrates (i.e. glass, plastic, type IV collagen, gelatin, fibronectin) are typically ineffective as substrates for cnidarian cells. Consequently, those substrates were not tested. Substrates that were tested included untreated Falcon Primaria plastic (a plastic that has been permanently modified to incorporate anionic and cationic functional groups) and Matrigel (BDBiosciences), a commercially available matrix. Neither was successful. We also tried to embed the cells in soft agar. This was moderately successful inasmuch as it immobilized the cells and they divided, but none extended processes. Cells were also plated onto fragments of mesoglea, the "jelly" of the jellyfish, prepared in a variety of ways. This substrate has been used successfully by other groups for short term culture, but in our hands, to date at least, long term culture is problematic due to bacterial contamination. We plan to explore ways to sterilize this substrate, without compromising its cell adhesion ability, when animals become available again.

Figure 1. Cells isolated from the base of *Chrysaora* tentacles and maintained in an artificial sea water culture medium, 25 days post-isolation.

Studies with cells isolated from *Cladonema* and *Physalia* yielded similar results; the tissues could be dissociated relatively easily and the isolated cells remained viable for extended periods of time, but did not attach to any of the substrates tested. This work will continue using lectins (see below) for cell attachment.

Lectin Binding

Lectins are complex, frequently plant-derived molecules that bind to specific sugar moieties on the surface of cell membranes and other biological structures. The strength and specificity of that binding is such that cell surface-specific lectins have been used to attach cells

to substrates for cell culture. While not part of the original plan of work, we carried out a lectin binding study with the goal of identifying means to immobilize and specifically orient isolated cysts in discharge devices, and to selectively isolate and culture interstitial cells.

Tentacles and dactylozooids from *Physalia* and *Cladonema* were fixed in 4%, phosphate-buffered paraformaldehyde, rinsed and permeabilized, then exposed to a battery of fluorescently-conjugated lectins (1:1000 dilution) overnight at 4 EC. After additional rinsing the tissues were examined using fluorescence microscopy and the presence and location of fluorescence noted.

A total of 20 lectins (Vector Labs.), which together target all the major sugar moieties, were screened. In the case of *Cladonema*, 6 lectins (GSL-2, Jacalin, VVA, DBA, LEL, and SBA) stained the cyst wall(Fig. 2A) and, of those, VVA produced particularly intense staining of the apical end of the cyst (Fig. 2B). A further four (WGA, DSL, PSL and STL) stained the apical membrane of the cnidocyte but not the cyst (Fig. 2C), and eight stained other cell types.

Fourteen lectins stained tissues from *Physalia*. Some, most notably Jacalin lectin, LEL, STL, GSL1, LCA and PSA, stained cysts to varying degree (Fig. 2D), but none preferentially stained the apical or basal ends of the cysts. WGA produced impressive staining of the sensory apparatus at the apical end of the intact cnidocyte (Fig. 2E) but not the cyst. Others, such as SBA, ECL, VVA and S-WGA stained other cells types in the dactylozooids(Fig. 2F) and tentacles.

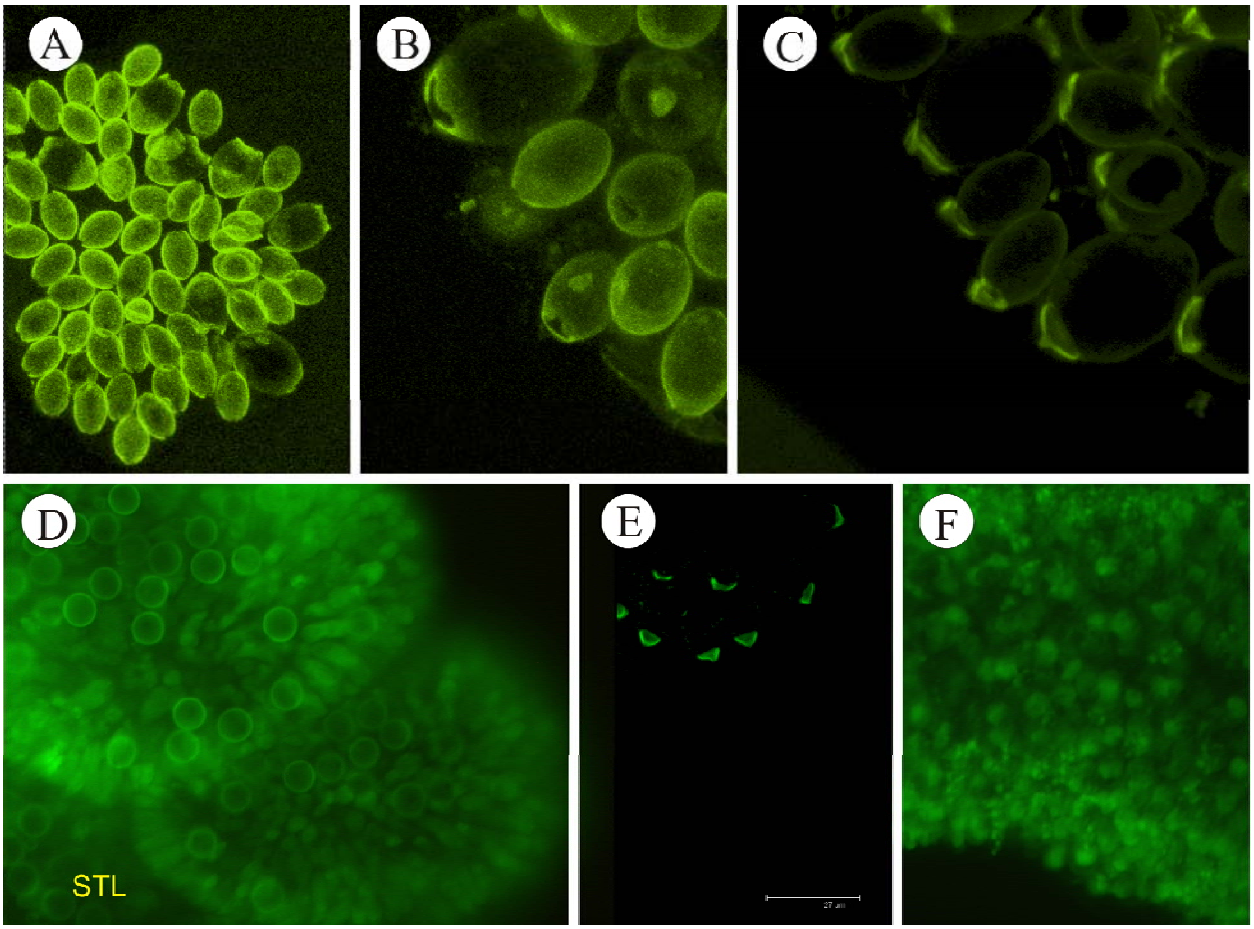

Figure 2. Lectin staining of tissues from *Cladonema* and *Physalia*. A. Cysts in the capitate bulb of *Cladonema* stained with fluorescein-conjugated DBA. B. The apical ends of cysts from *Cladonema* stained with VVA. C. WGA staining of the apical membrane and sensory apparatus of cnidocytes from *Cladonema*. D. STL staining of

cysts in tentacles of *Physalia*. E. WGA staining of the sensory apparatus in the apical membrane of *Physalia* cnidocytes. F. Putative interstitial cells in a dactylozzod stained with VVA.

Future Directions.

Progress on the goal of characterizing the transcriptome of developing cnidocytes was limited by the quality of the cDNA libraries obtained and, specifically, the amount of ribosomal RNA present. The quality of the latest library is much improve and, while not ideal, is sufficient to provide the necessary information. Future work will, therefore, focus on obtaining large amounts of sequence information, creating good assemblies of those sequences, and annotating and cataloging the genes. We will also conduct a study of non-coding RNAs since they will likely be important regulators of gene expression.

We will also explore the feasibility of using the results of the lectin binding study describe above to identify possible substrates for cell adhesion.